APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2000, p. 3981–3986 Vol. 66, No. 9
0099-2240/00/$04.00+0

Characteristics of *Vibrio parahaemolyticus* O3:K6 from Asia

HIN-CHUNG WONG,[1]* SHU-HUI LIU,[1] TIEN-KUEI WANG,[2] CHIH-LUNG LEE,[2] CHIEN-SHUN CHIOU,[2] DING-PING LIU,[3] MITSUAKI NISHIBUCHI,[4] AND BOK-KWON LEE[5]

Department of Microbiology, Soochow University, Taipei, Taiwan 111,[1] and Bacteriology Division[2] and Virology Division,[3] Center for Disease Control, Taipei, Taiwan 115, Republic of China; Center for Southeast Asian Studies, Kyoto University, Kyoto 606-8501, Japan[4]; and Laboratory of Enteric Infection, Department of Microbiology, National Institute of Health, Seoul, Korea[5]

Received 14 October 1999/Accepted 6 July 2000

A variety of serovars of the food-borne pathogen *Vibrio parahaemolyticus* normally cause infection. Since 1996, the O3:K6 strains of this pathogen have caused pandemics in many Asian countries, including Taiwan. For a better understanding of these pandemic strains, the recently isolated clinical O3:K6 strains from India, Japan, Korea, and Taiwan were examined in terms of pulsed-field gel electrophoresis (PFGE) typing and other biological characteristics. After PFGE and cluster analysis, all the O3:K6 strains were grouped into two unrelated groups. The recently isolated O3:K6 strains were all in one group, consisting of eight closely related patterns, with I1(81%) and I5(13%) being the most frequent patterns. Pattern I1 was the major one for strains from Japan, Korea, and Taiwan. All recently isolated O3:K6 strains carried the thermostable direct hemolysin (*tdh*) gene. No significant difference was observed between recently isolated O3:K6 strains and either non-O3:K6 reference strains or old O3:K6 strains isolated before 1996 with respect to antibiotic susceptibility, the level of thermostable direct hemolysin, and the susceptibility to environmental stresses. Results in this study confirmed that the recently isolated O3:K6 strains of *V. parahaemolyticus* are genetically close to each other, while the other biological traits examined were usually strain dependent, and no unique trait was found in the recently isolated O3:K6 strains.

Vibrio parahaemolyticus, a common food-borne enteric pathogen in Asia, causes approximately half of the food poisoning outbreaks in Taiwan, Japan, and several Southeast Asian countries (5, 11). Clinical manifestations of *V. parahaemolyticus* infections include diarrhea, abdominal cramps, nausea, vomiting, headaches, fever, and chills, with the incubation period ranging from 4 to 96 h (11). Most clinical strains of *V. parahaemolyticus* produce a major virulence factor, the thermostable direct hemolysin (TDH), and are designated as Kanagawa phenomenon positive (KP^+). Another virulence factor, the TDH-related hemolysin (TRH), is generally associated with the KP^- (urease positive) strains of *V. parahaemolyticus* (13). The KP^- strains are also involved in some food poisoning outbreaks (8) and are seen sporadically in wound infections (10).

Isolates of *V. parahaemolyticus* can be differentiated from each other by serotyping; 13 O groups and 71 K types have already been identified (9). Although diversified serovars normally cause infections, a special serovar, O3:K6, abruptly appeared in India in 1996. These O3:K6 serovar organisms carrying the *tdh* gene accounted for 50 to 80% of *V. parahaemolyticus* infections in Calcutta after February 1996. Strains belonging to the same group were isolated from travelers arriving in Japan from various Southeast Asian countries (19). In Taiwan, O1:K56, O3:K29, O4:K8, and O5:K15 were the most frequently isolated serovars from 1992 to 1995, though no serovar was dominant. Since 1996, however, the O3:K6 strains have caused numerous outbreaks in Taiwan, accounting for 51, 79, 61, and 65% of the outbreaks in 1996, 1997, 1998, and the first half of 1999, respectively (unpublished data). This strain may also cause a pandemic spread to other continents. During July to September 1998, an outbreak of O3:K6 *V. parahaemolyticus* infections associated with consumption of oysters and clams harvested from Long Island Sound occurred among residents of Connecticut, New Jersey, and New York. Laboratory testing of 12 *V. parahaemolyticus* clinical isolates, including the 8 traced to Oyster Bay, identified the O3:K6 serotype, which had not previously been detected in coastal waters of the United States. It is possible that this strain was introduced to U.S. coastal waters by ballast water discharged from ships which had traveled to Asia (3, 4).

In this study, we collected 139 isolates of O3:K6 *V. parahaemolyticus* strains recently isolated in Taiwan. Some of these strains were isolated from travelers originating in several other Asian countries. The clonal relationship of these strains to the O3:K6 strains isolated in other Asian countries was analyzed by the pulsed-field gel electrophoresis (PFGE) method (25). Biological characteristics, such as high toxin productivity or ability to survive in the natural environment, that are unique to the pandemic strains might provide further insight into the mechanism of the emergence and spread of these strains. Resistance to antibiotics and to environmental stresses, such as low- or high-temperature inactivation or a mild acid or low-salinity treatment, may enhance the survival rates and spread of these O3:K6 strains in the host and the environment. The recently isolated O3:K6 strains were also compared to O3:K6 strains isolated before 1996 and to non-O3:K6 strains for the presence and expression of the *tdh* gene encoding TDH and for susceptibilities to antibiotics and various environmental stresses.

MATERIALS AND METHODS

Bacterial cultures. The clinical stool specimens were collected from patients in food poisoning outbreaks, transported to the laboratory, and analyzed in less than 8 h according to standard procedures (6). The specimens were added to 100 ml of alkaline peptone water and incubated at 37°C for 7 to 8 h. A loopful of the enrichment culture was streaked on thiosulfate-citrate-bile salt-sucrose agar (Difco Laboratories, Detroit, Mich.) and incubated at 37°C for 18 to 24 h. The

* Corresponding author. Mailing address: Department of Microbiology, Soochow University, Taipei, Taiwan 111, Republic of China. Phone: (886) 2-28819471, ext. 6852. Fax: (886) 2-28831193. E-mail: wonghc@mbm1.scu.edu.tw.

TABLE 1. The toxin production and susceptibilities to antibiotics and environmental stresses of *V. parahaemolyticus*[a]

Strain no.	Serotype	City or origin of travelers	Country of isolation	Date of isolation (yr/mo/day)	PFGE pattern	TDH titer	*tdh* gene	*D* value at pH4 (min)	D_{50C}	% Survival at 4°C (day 6)	*D* value at 0.2% NaCl (min)	Antibiogram pattern
166	O3:K29	Taichung	Taiwan	1992/10/05	A5	512	+	6.96	3.20	10.99	100	9
272	O3:K6	Kaohsiung	Taiwan	1993/01/04	B2	ND	ND	ND	ND	ND	ND	ND
314	O3:K29	Miao-Li	Taiwan	1993/06/14	A6	2,048	+	4.23	4.94	12.01	92	27
556	O3:K29	Taipei	Taiwan	UN	C5	512	+	7.93	6.57	19.39	78	21
620	O5:K15	Kaohsiung	Taiwan	1994/04/16	B1	1,024	+	6.82	2.64	13.19	40	1
638	O5:K15	Kaohsiung	Taiwan	1994/04/16	B1	2,048	+	9.45	5.80	6.36	67	28
665	O1:K56	Chia-i	Taiwan	1994/06/13	C6	1,024	+	11.41	3.44	6.84	74	28
667	O1:K56	Chia-i	Taiwan	1994/06/13	C6	256	+	5.68	2.62	18.12	49	39
669	O1:K56	Chia-i	Taiwan	1994/6/13	B1	1,024	+	5.41	3.85	13.45	69	25
675	O4:K8	Taipei	Taiwan	1994/6/30	D3	ND	+	ND	ND	ND	ND	ND
676	O4:K8	Taipei	Taiwan	1994/6/30	D3	512	+	ND	ND	ND	ND	ND
680	O4:K8	P'eng-Hu	Taiwan	1994/6/30	D3	1,024	+	9.32	5.16	6.87	63	14
701	O5:K15	Kaohsiung	Taiwan	1994/07/28	Q	256	+	5.15	2.43	12.21	183	2
736	O4:K8	P'ing-Tung	Taiwan	1994/9/17	D4	256	+	8.59	4.28	9.78	65	16
1020	O3:K6	Phillipines	Taiwan	1997/05/05	I5	ND	+	6.72	3.28	4.43	66	15
1021	O3:K6	Singapore	Taiwan	1997/07/14	I6	ND	+	4.91	5.37	11.29	68	36
1077	O3:K6	Miao-Li	Taiwan	1997/04/25	I5	256	+	7.13	3.01	17.14	78	3
1078	O3:K6	Miao-Li	Taiwan	1997/04/25	I1	256	+	6.40	2.63	15.02	71	38
1084	O3:K6	Changh	Taiwan	1997/04/21	I7	512	+	6.97	4.82	18.10	71	29
1091	O3:K6	Taichung	Taiwan	1997/04/29	I1	512	+	4.16	3.28	14.89	74	4
1092	O3:K6	Taichung	Taiwan	1997/05/13	I5	256	+	5.28	3.42	5.68	66	17
1114	O3:K6	Miao-Li	Taiwan	1997/06/16	ND	0	+	7.01	3.81	7.42	64	8
1115	O3:K6	Miao-Li	Taiwan	1997/06/16	ND	32	+	5.22	3.09	7.14	66	18
1121	O3:K6	Miao-Li	Taiwan	1997/06/25	I1	1,024	ND	7.12	2.79	16.10	129	37
1123	O3:K6	Miao-Li	Taiwan	1997/06/28	ND	256	+	5.37	7.34	16.39	73	12
1126	O3:K6	Taichung	Taiwan	1997/06/30	ND	128	ND	ND	ND	ND	ND	ND
1127	O3:K6	Taichung	Taiwan	1997/06/30	ND	128	ND	ND	ND	ND	ND	ND
1129	O3:K6	Yun-Lin	Taiwan	1997/07/16	ND	1,024	+	4.26	3.00	11.13	120	20
1130	O3:K6	Yun-Lin	Taiwan	1997/07/16	ND	1,024	+	4.64	9.91	17.54	128	29
1132	O3:K6	Yun-Lin	Taiwan	1997/07/22	I1	512	+	4.69	3.65	7.55	70	19
1134	O3:K6	Taichung	Taiwan	1997/08/17	I1	512	ND	ND	ND	ND	ND	ND
1137	O3:K6	Yun-Lin	Taiwan	1997/09/07	I5	256	+	5.91	5.45	7.26	76	5
1139	O3:K6	Yun-Lin	Taiwan	1997/09/07	I4	256	ND	ND	ND	ND	ND	ND
1147	O3:K6	Changhua	Taiwan	1997/10/05	I1	512	+	11.09	3.40	11.42	74	17
1154	O3:K6	Taichung	Taiwan	1997/11/11	I2	256	+	7.33	3.77	5.90	83	11
1159	O3:K6	Changhua	Taiwan	1997/11/21	ND	256	+	19.49	3.83	15.92	56	35
1188	O3:K6	Hua-lien	Taiwan	1998	ND	128	+	5.74	3.18	7.12	71	19
1189	O3:K6	Thailand	Taiwan	1998	I1	ND	+	13.65	5.89	11.11	67	30
1222	O3:K6	Hua-lien	Taiwan	1998	ND	ND	ND	ND	ND	ND	ND	23
1223	O3:K6	Hua-lien	Taiwan	1998	ND	ND	ND	ND	ND	ND	ND	33
1224	O3:K6	Hua-lien	Taiwan	1998	ND	ND	ND	ND	ND	ND	ND	22
1225	O3:K6	Hua-lien	Taiwan	1998	ND	ND	ND	ND	ND	ND	ND	24
1226	O3:K6	Hua-lien	Taiwan	1998	ND	ND	ND	ND	ND	ND	ND	26
1227	O3:K6	Hua-lien	Taiwan	1998	ND	ND	ND	ND	ND	ND	ND	33
1228	O3:K6	Hua-lien	Taiwan	1998	ND	ND	ND	ND	ND	ND	ND	24
1229	O3:K6	Hua-lien	Taiwan	1998	ND	ND	ND	ND	ND	ND	ND	34
1230	O3:K6	Hua-lien	Taiwan	1998	ND	ND	ND	ND	ND	ND	ND	24
VP47	O3:K6	Calcutta	India	1996	I1	ND	ND	ND	ND	ND	ND	31
VP138	O3:K6	Calcutta	India	1996	I5	ND	ND	ND	ND	ND	ND	31
KX-V224	O3:K6	Thailand	Japan	1996	I1	ND	ND	ND	ND	ND	ND	32
KX-V225	O3:K6	Thailand	Japan	1996	I1	ND	ND	ND	ND	ND	ND	31
KX-V226	O3:K6	Singapore	Japan	1996	I1	ND	ND	ND	ND	ND	ND	31
KX-V231	O3:K6	Thailand	Japan	1996	I1	ND	ND	ND	ND	ND	ND	31
AQ3732	O3:K6	UN	Japan	1982	A3	ND	ND	ND	ND	ND	ND	ND
AQ3794	O3:K6	Singapore	Japan	1983	A3	ND	ND	9.84	3.36	16.52	65	3
AQ3810	O3:K6	Singapore	Japan	1983	R	ND	ND	ND	ND	ND	ND	ND
AQ4019	O3:K6	Maldive Islands	Japan	1985	A3	ND	ND	6.29	2.77	8.28	66	20
AQ4235	O3:K6	Thailand	Japan	1987	A1	ND	ND	6.54	3.12	6.08	73	27
AQ4644	O3:K6	Thailand	Japan	1991	A3	ND	ND	5.56	2.40	16.16	98	32
AQ4733	O3:K6	Singapore	Japan	1992	A2	ND	ND	ND	ND	ND	ND	ND
AQ4853	O3:K6	Hong Kong	Japan	1993	A3	ND	ND	3.56	3.74	6.71	56	12
AQ4901	O3:K6	Thailand	Japan	1993	A3	ND	ND	4.25	2.53	10.15	66	38
KX-V138	O3:K6	Indonesia	Japan	1995	ND	ND	ND	5.31	4.29	6.30	125	7
97-804	O3:K6	Jeju	Korea	1997/07/19	I3	128	+	6.12	2.91	4.72	69	13
97-1205	O3:K6	Kyung-Nam	Korea	1997/09/08	I1	256	+	5.45	2.85	9.79	144	20
97-1576	O3:K6	Gyung-Gi	Korea	1997/08/27	I8	1,024	+	4.72	3.62	10.97	69	6
97-1640	O3:K6	Kwang Won	Korea	1997/10/10	I1	2,048	+	8.69	2.42	10.69	44	10
98-39	O3:K6	Jun-Nam	Korea	1998/07/10	I1	ND	+	ND	ND	ND	ND	ND

[a] For comparison with recently identified O3:K6 strains isolated after 1996, the O3:K6 strains and other frequently occurring serovars isolated before 1996 were selected and examined in this study. UN, unknown; ND, not determined; D_{50C}, *D* value at 50°C. Toxin production and the presence of toxin genes in strains from India and Japan have been previously reported (19).

TABLE 2. PFGE typing of O3:K6 strains of *V. parahaemolyticus* isolated before and after 1996

PFGE pattern[a]	No. of isolate (%)	Origin of isolate (*n*)[b]	Year(s) isolated	Origin of travelers (*n*)[c]
Before 1996				
A1	2	Japan (2)	1987	Thailand (2)
A2	1	Japan (1)	1992	Singapore (1)
A3	9	Japan (9)	1982–1993	Unknown (1), Singapore (1), Hong Kong (2), Maldive Islands (3), Thailand (2)
B2	1	Taiwan (1)	1993	
R	1	Japan (1)	1983	Singapore (1)
After 1996				
I1	155 (81.2)	Korea (15)	1998	
		India (1)	1996	
		Japan (4)	1996	Singapore (1), Thailand (3)
		Taiwan (135)	1997	Thailand (2), Philippines (2)
I2	1 (0.5)	Taiwan (1)	1997	
I3	3 (1.6)	Korea (1)	1998	
		Taiwan (2)	1997	Thailand (1)
I4	1 (0.5)	Taiwan (1)	1997	
I5	25 (13.1)	Korea (1)	1998	
		India (1)	1996	
		Taiwan (23)	1997	Philippines (2)
I6	2 (1.0)	Taiwan (2)	1997	Indonesia (1), Singapore (1)
I7	1 (0.5)	Taiwan (1)	1997	
I8	3 (1.6)	Korea (1)	1998	
		Taiwan (2)	1997	

[a] Designation of these patterns followed a revised PFGE typing scheme for the clinical strains collected between 1992 and 1995 in our laboratory.
[b] *n*, number of isolates.
[c] Some strains were isolated from travelers returning from other Asian countries to the place of the origin of the isolates; others are domestic strains. *n*, number of strains.

bacterial colonies were randomly selected and subjected to species identification using API 20E identification strips (API Systems, Montalieu-Vercieu, France) and by conventional methods (22). The isolates were serotyped by using commercial antisera (Denka Seiken, Tokyo, Japan). The cultures were stored at −85°C in tryptic soy broth (Difco)–3% NaCl containing 20% glycerol.

Fourteen and 191 strains of O3:K6 *V. parahaemolyticus* isolated before and after 1996, respectively, were analyzed by PFGE. One hundred sixty-eight strains were isolated in Taiwan from different food poisoning outbreaks, and 2, 17, and 18 clinical strains were obtained from India, Japan, and Korea, respectively (Tables 1 and 2). The TDH production, antibiotic susceptibility, and susceptibility to environmental stresses of these O3:K6 strains were compared to those for 13 other clinical non-O3:K6 but frequently isolated serovars collected before 1996 in Taiwan (Table 1).

Determination of TDH titer. Each test strain was cultured in a broth medium composed of 2% Bacto Peptone, 0.5% D-mannitol, and 5% NaCl (pH 7.8) with shaking (180 rpm) at 37°C for 16 h. Twofold dilutions were made by using uninoculated culture broth in a 96-well microplate. The TDH titer in the spent culture medium was determined with reversed passive latex agglutination kits (Denka Seiken) (24).

Determination of antibiotic susceptibility. Susceptibility of each test strain to antibiotics was examined by the disk diffusion method (23). Antibiotic-loaded paper disks (Difco) were dispensed on Mueller-Hinton agar plates with bacterial lawn. After incubation at 37°C for 14 to 18 h, the size of the inhibition zone was recorded and interpreted according to the reference provided by the manufacturer. Twelve antibiotic disks were used: ampicillin (10 μg), cephalothin (30 μg), colistin (10 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), rifampin (5 μg), streptomycin (10 μg), tetracycline (30 μg), tobramycin (10 μg), and vancomycin (30 μg).

Detection of *tdh* by PCR. The presence of the *tdh* gene in the test strain was examined by PCR with the use of primers 5′-GTACCGATATTTTGCAAA-3′ and 5′-ATGTTGAAGCTGTACTTGA-3′, which were synthesized according to the published *tdh* nucleotide sequence (18), followed by detection of a 382-bp amplified fragment sequence.

The isolate was cultured on nutrient agar (Difco)–1% NaCl medium at 37°C overnight. Several well-grown colonies were chosen and resuspended in 300 μl of TEB buffer containing 10 mM Tris-HCl, 1 mM EDTA disodium salt, and 0.1% sodium dodecyl sulfate, heated at 45°C for 30 min to lyse the cells. The lysate was centrifuged, and the supernatant was stocked at −20°C. DNA amplifications were performed in a reaction mixture consisting of a buffer solution (10 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl [pH 8.3]) containing 200 μM (each) dATP, dCTP, dGTP, and dTTP, 10 μM primer, 0.15 μl of DyNAZyme II thermostable DNA polymerase (Finnzymes Oy, Espoo, Finland), and 3 μl of the lysate DNA in a final volume of 30 μl. Amplification was performed in a thermal cycler (Personal cycler 20; Biometra Biomedizinische Analytik GmbH, Göttingen, Germany). The reaction mixture was overlaid with 50 μl of sterile mineral oil and then was incubated in a thermal cycler at 95°C for 5 min. Thermostable DNA polymerase was added, and amplification was carried out for 40 cycles, each of which was set as follows: 94°C for 1 min, 48°C for 1 min, 72°C for 1 min, and finally, an additional 72°C for 5 min. The amplicons were detected by 1.8% agarose gel electrophoresis.

PFGE. DNA extraction, DNA digestion, and PFGE were performed according to procedures described elsewhere (25). Bacteria on tryptic soy agar (Difco)–3% NaCl were transferred to 5 ml of TSB–3% NaCl and incubated overnight at 37°C with shaking at 160 rpm. Bacterial cells were harvested by centrifugation and resuspended in 2 ml of buffer containing 10 mM Tris, 100 mM EDTA, and 1 mM NaCl at pH 8.0. Agarose plugs were prepared by mixing an equal volume of bacterial suspension with 1.5% low-melting agarose (FMC Corp., Rockland, Maine). Bacterial cells in the agarose plugs were lysed by treatment with a solution containing 1 mg of lysozyme/ml and 0.1% *N*-sodium lauroyl sarcosine at 37°C for 24 h. The cells were then treated with proteinase K (0.5 mg/ml in 0.5 M EDTA and 1% *N*-sodium lauroyl sarcosine) at 45°C for 48 h and washed three times (30 min each) with TE buffer (10 mM Tris-HCl, 1 mM EDTA). One section of the plug (4 by 9 by 1.2 mm) was equilibrated with an enzyme buffer and then placed in 100 μl of fresh buffer containing 10 U of *Sfi*I (New England BioLabs, Beverly, Mass.). It was then incubated at 4°C for 16 h and, finally, digestion was performed at 37°C for another 48 h.

High-molecular-weight restriction fragments were resolved in 1% agarose gel in 0.5% Tris-borate-EDTA buffer by using a CHEF apparatus (CHEF-DR II; Bio-Rad Laboratories, Richmond, Calif.). The running conditions were 190 V for 22.4 h at 14°C, with 3 to 80 s of pulse time. A lambda ladder PFGE marker (New England BioLabs) was used to mark molecular size. After electrophoresis was performed, gels were stained in ethidium bromide (Sigma Co., St. Louis, Mo.), destained in distilled water, and photographed with a UV transilluminator, the Flou-Link 312 (Vilber Lourmat, Torey, France).

Susceptibility to environmental stresses. To determine the susceptibility to different environmental stresses, i.e., temperature or conditions of mild acid or low salinity, bacteria were cultured in 50 ml of Luria-Bertani broth–3% NaCl medium at 37°C for 16 h. *V. parahaemolyticus* is a vulnerable species and will be quickly inactivated under conditions that differ from those of its natural habitat in warm marine water (12). Normally, this bacterium lives and proliferates in warm seawater, with optimum growth at 35 to 37°C, 3% NaCl, and neutral acidity (2). The maximum growth range of this pathogen is about 5 to 44°C, pH 4.8 to 11.0 (2). Temperature at 13°C or salinity below 0.5% NaCl inhibits growth of this bacterium (2). Although *V. parahaemolyticus* is alkaline tolerant, treatment with mild acid at pH 4.4 is lethal (26). For temperature stresses, the cultures were shifted to either 4 or 50°C (14). For mild acid stress, the bacterial cultures were

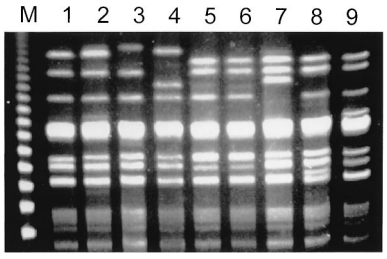

FIG. 1. PFGE patterns of recently isolated O3:K6 strains of *V. parahaemolyticus*. Conditions for PFGE were 1% agarose gel, 0.5× Tris-borate-EDTA buffer, 190 V, pulse time 3 to 80 s, for 22.4 h. Lane 1, isolate 1020 (from Philippines; pattern I5); lane 2, isolate 1021 (from Singapore; pattern I6); lane 3, isolate 1084 (from Taiwan; pattern I7); lane 4, isolate 1104 (from Taiwan; pattern I8); lane 5, isolate 1123 (from Taiwan; pattern I1); lane 6, isolate 1125 (from Taiwan; pattern I1); lane 7, isolate 1139 (from Taiwan; pattern I4); lane 8, isolate 1154 (from Taiwan; pattern I2); lane 9, isolate 97-804 (from Korea; pattern I3); lane M, lambda ladder PFGE marker.

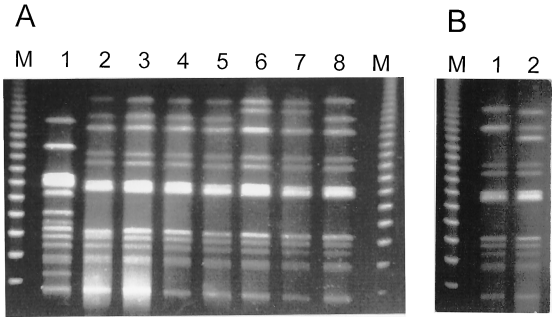

FIG. 2. PFGE patterns of O3:K6 strains of *V. parahaemolyticus* isolated before 1996. (A) Lane 1, isolate AQ3810 (traveler from Singapore; pattern R); lane 2, isolate AQ4019 (traveler from Singapore; pattern A3); lane 3, isolate AQ4037 (traveler from Maldive Islands; pattern A3); lane 4, isolate AQ4093 (traveler from Maldive Islands; pattern A3); lane 5, isolate AQ4133 (traveler from Hong Kong, pattern A3); lane 6, isolate AQ4235 (traveler from Thailand; pattern A1); lane 7, isolate AQ4299 (traveler from Thailand; pattern A1); lane 8, isolate AQ4644 (traveler from Hong Kong; pattern A3); lane M, lambda ladder PFGE marker, 48.5 kb at the bottom with an increment of 48.5 kb. (B) Lane 1, isolate AQ4733 (traveler from Singapore; pattern A2); lane 2, isolate AQ4853 (traveler from Hong Kong; pattern A3); lane M, lambda ladder PFGE marker.

acidified to pH 4.0 by adding 12 N HCl. For low-salinity stress, the bacterial cells were collected by centrifugation, washed, and resuspended in 50 ml of 0.2% NaCl (26). After shifting to different stress conditions, the numbers of surviving cells were determined at various intervals by the dilution plate count method. Decimal dilutions were prepared in 0.1% peptone–3% NaCl, and the diluted cultures were plated on tryptic soy agar–3% NaCl and incubated at 37°C overnight. Triplicate determinations were performed for each dilution. Finally, the *D* values (the time required to cause a 90% reduction in the count of viable cells) for 50°C, pH 4, and low salinity were calculated by the curve-fit function of Slide Write Plus software, version 1.10 (Advanced Graphic Software, Inc., Carlsbad, Calif.). The percent survival was also determined for each strain at 4°C for 6 days when a significant decrease of survivors was observed.

Statistical analysis. The size of each band resolved in PFGE was determined by Stratascan 7000 densitometry with one-dimensional analysis software (Stratagene, La Jolla, Calif.). Data were coded as 0 (negative) or 1 (positive). Hierarchical cluster analysis was performed using the average linkage method with the squared Euclidean distance measure (15). The dendrogram was produced with the SPSS for Windows Release 6.0 program (SPSS, Inc., Chicago, Ill.). The susceptibilities to environmental stresses for different categories of *V. parahaemolyticus* strains were examined by analysis of variance with Duncan's multiple range test at $P < 0.05$.

RESULTS AND DISCUSSION

PFGE analysis. In contrast to *V. cholerae*, infections of *V. parahaemolyticus* were usually not associated with particular dominating serovars in the past. In this study, we examined 205 strains of O3:K6 *V. parahaemolyticus* by PFGE following the digestion of the *Sfi*I enzyme. Most of these strains were isolated in Taiwan, Korea, and Japan, with a portion of Taiwanese strains and all Japanese strains isolated from travelers originating in other Asian countries. Those strains accurately represent the pandemic strains in Asia. Cumulatively, 13 different PFGE patterns were discriminated in these O3:K6 strains (Tables 1 and 2 and Fig. 1 and 2). After cluster analysis, these PFGE patterns appeared to be divided into two distinct groups (Fig. 3). Designation of these patterns followed a revised PFGE typing scheme for the clinical strains collected between 1992 and 1995 in our laboratory. Those strains isolated before 1996 belonged to one group, with patterns A1, A2, A3, B2, and R. This group was also not homogenous with two satellite patterns B2 and R. The strain 272 with pattern B2 was isolated in 1993 in Kaohsiung, Taiwan, while strain AQ3810 (pattern R) was isolated from a traveler returning to Japan from Singapore in 1983. Patterns A1, A2, and A3 were also genetically closely related to each other (Table 1 and Fig. 2). Those recently identified O3:K6 strains isolated after 1996 and causing pandemics were grouped into eight closely related PFGE patterns, I1 to I8, with pattern I1 (81.2%) the most frequently isolated pattern, followed by pattern I5 (13.1%). Pattern I1 was also the major one for strains from different countries, such as Taiwan, Korea, Japan, and India (Table 2). Notably, patterns I3, I5, and I8 were found in strains isolated from Taiwan and several other countries. Moreover, they are probably dispersed in this pandemic spread along with the major I1 pattern. Genomic reassortment has been demonstrated by Bag et al. (1) when examining the O3:K6 strains of India. It is speculated that these minor patterns (I2 to I8) are derived from the I1 pattern after minor genomic reassortment, although we needed more confirmative genetic study. Another interesting finding is that the nine strains of pattern A3 were isolated from travelers to Japan returning from different countries, such as Singapore, Hong Kong, Thailand, and the Maldive Islands, between 1982 and 1993 (19) (Table 2). The closely related patterns A1, A2, and A3 indicated that before the incidence of O3:K6 strains in 1996, spreading of genetically similar clones of *V. parahaemolyticus* had occurred in these Asian countries for many years.

***tdh* detection and TDH production.** J. Okuda et al. also reported that the O3:K6 strains recently isolated in India and Japan were *tdh* positive and *trh1* and *trh2* negative. The levels of TDH production in these strains did not differ significantly from those of other KP^+ strains carrying the *tdh* gene (19). The

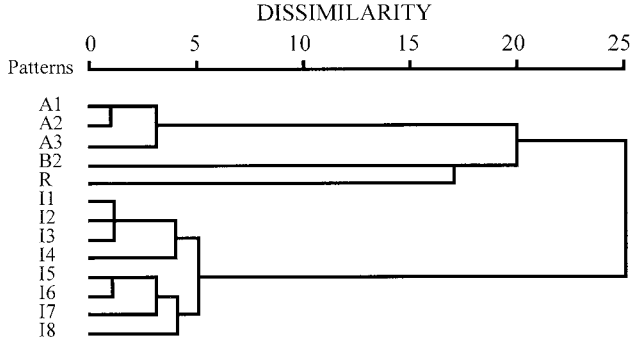

FIG. 3. Dendrogram showing the clustering of PFGE patterns for O3:K6 strains of *V. parahaemolyticus*. The dendrogram was based on the squared Euclidean distance measure and average linkage clustering method by the SPSS for Windows Release 6.0 program. The dissimilarity units are arbitrary and are based on the sum of the squared presence of characters.

TABLE 3. Antibiotic susceptibilities of O3:K6 and other strains of *V. parahaemolyticus* isolated from cases of food poisoning

Antibiotic (amt [μg])	% Resistant[a]	
	Recent O3:K6 strains[b] (n = 39)	Other strains[c] (n = 18)
Ampicillin (10)	97.4	100
Cephalothin (30)	56.4	66.7
Colistin (10)	51.3	38.9
Erythromycin (15)	48.7	72.2
Gentamicin (10)	23.1	44.4
Kanamycin (30)	15.4	22.2
Nalidixic acid (30)	0.0	5.6
Rifampin (5)	69.2	62.5
Streptomycin (10)	41.0	38.9
Tetracycline (30)	10.3	5.6
Tobramycin (10)	23.1	27.8
Vancomycin (30)	92.3	88.9

[a] The percentages of resistant strains are listed; others are sensitive or intermediately susceptible to these antibiotics.
[b] O3:K6 strains isolated after 1996. *n*, number of strains.
[c] Other *V. parahaemolyticus* strains, including O3:K6 strains and those frequently isolated serovars isolated in Taiwan and Japan before 1996.

tdh gene was detected by PCR in all of the 37 selected strains: 24 were O3:K6 strains recently isolated in Taiwan and Korea and 13 were other non-O3:K6 reference strains (Table 1).

We compared the levels of TDH production for 25 O3:K6 strains recently isolated in Korea and Taiwan to those for 12 other *tdh* gene-positive strains isolated before 1996 (Table 1). We confirmed that O3:K6 strains recently isolated in Taiwan and Korea did not produce TDH at significantly higher levels than did the strains isolated before 1996. Although an O3:K6 strain recently isolated in Taiwan, strain 1114, was *tdh* positive, the TDH production in this strain was not detected. This may be due to a mutation in the promoter of the *tdh* gene (20).

Susceptibility to antibiotics. Strains of *V. cholerae* isolated after the epidemic of serovar O139 reveal an expanding antibiotic resistance to a variety of drugs compared to the O1 strains isolated before the advent of the O139 serovar (17). Enhanced antibiotic resistance of pathogens may also increase their survival rates. In this study, 55 strains isolated before and after 1996 were examined for their susceptibilities to 12 antibiotics by the disk diffusion method. Most of these strains were resistant to ampicillin and vancomycin, and 56.4, 48.7, and 69.2% of these strains were resistant to cephalothin, erythromycin, and rifampin, respectively (Table 3). The recently isolated O3:K6 strains were compared with O3:K6 and non-O3:K6 strains isolated before 1996, revealing that these two groups are similar in antibiotic susceptibility. The O3:K6 strains recently isolated in India were judged to be generally sensitive to eight antibiotics, four of which were tested in this study, when their MICs were determined (19). A comparison of the results obtained in the two studies indicated that sensitivities of the Indian strains and the recently isolated O3:K6 strains examined in this study were similar.

The patterns of antibiotic susceptibility for those strains examined were grouped into 39 different antibiograms (data not shown). In contrast to the PFGE analysis, the distribution of different antibiograms was not associated with specific serovar or PFGE patterns (Table 1). Antibiogram analysis is a useful typing method for some pathogens (21). However, antibiotic susceptibility is a changeable phenotypic trait compared to the stable results of PFGE analysis of the chromosomal DNA (21). The presence of the R plasmid in this pathogen has been shown and will probably enhance the variation of antibiotic susceptibility in different strains (7). So the finding of various antibiograms among these O3:K6 strains was a plausible result.

Susceptibility to environmental stresses. Bacteria that are highly resistant to environmental stress may survive better in the environmental substrate and have a greater chance to spread. Thirty of the recently isolated O3:K6 strains from Taiwan, Korea, and Japan were examined for their susceptibilities to different environmental stresses and compared to those of other reference strains (Table 1). The average *D* values at 50°C, at pH 4, and at 0.2% NaCl were about 3 to 4 min, 6 to 11 min, and 80 to 120 min for different categories of strains, respectively. About 5 to 12% of the strains of O3:K6 and non-O3:K6 serovars isolated before 1996 survived at 4°C for 6 days (Table 4). No particularly stress-resistant strain was discerned. *V. parahaemolyticus* is a vulnerable species and is usually rapidly inactivated at 48 and 55°C, at salinity below 0.5%, or at pH 4.0 (2). When the strains were grouped according to their location of isolation and compared, some groups differed significantly. For example, the recently isolated O3:K6 strains from Japan had a significantly lower low-temperature survival rate while having a higher resistance to mild acid and low-salinity treatments. When all the recently isolated O3:K6 strains as a group were compared with other strains, the group of recently isolated O3:K6 strains basically did not show any specific trait that would enhance its survival in the environ-

TABLE 4. Comparison of the O3:K6 strains isolated after 1996 and the O3:K6 and non-O3:K6 strains of *V. parahaemolyticus* isolated before 1996

Strain group (n)[a]	D_{50C} (min)[b]		% Survival at 4°C		*D* value at pH 4 (min)		*D* value 0.2% NaCl (min)	
	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE
Recent O3:K6 strains (30)[c]								
Japanese strains (6)	2.47–4.28	3.44 ± 0.25 a	0.36–11.23	5.27 ± 2.00 b	7.76–15.60	10.51 ± 1.27 a	66–194	119 ± 18 a
Korean strains (4)	2.42–3.62	2.95 ± 0.25 a	4.72–10.97	9.04 ± 1.46 ab	4.72–8.69	6.25 ± 0.86 b	44–144	82 ± 22 b
Taiwanese strains (20)	2.63–9.91	4.26 ± 0.40 a	4.43–18.10	11.43 ± 1.03 a	4.16–19.49	7.15 ± 0.83 ab	56–129	79 ± 5 b
Other strains (18)[d]								
Japanese strains (7)	2.40–4.29	3.17 ± 0.26 a	6.08–16.52	10.03 ± 1.71 ab	3.56–9.84	5.91 ± 0.77 b	56–125	78 ± 9 b
Taiwanese strains (11)	2.43–6.57	4.08 ± 0.42 a	6.36–19.39	11.75 ± 1.30 a	4.23–11.41	7.36 ± 0.66 ab	40–183	80 ± 12 b

[a] *n*, number of strains.
[b] D_{50C}, *D* value at 50°C.
[c] Recently isolated O3:K6 strains analyzed were obtained from Japan, Korea, and Taiwan after 1996.
[d] Other strains included O3:K6 and non-O3:K6 strains isolated in Japan and Taiwan before 1996. Means followed by the same letter were not significantly different by analysis of variance with Duncan's multiple range test at $P < 0.05$.

ment. There should be undiscovered characteristics in these O3:K6 strains that enable their pandemic spread.

As compared by the arbitrarily primed PCR method, the O3:K6 strains isolated between 1982 and 1993 differed from those isolated after 1996; those recently isolated O3:K6 strains belonged to a unique clone (19). Clonality of the new O3:K6 strains was also confirmed by the analysis of the *toxRS* sequence, which differs from those of the old O3:K6 strains at at least seven base positions within a 1,346-bp region. A new PCR method targeted for the *toxRS* sequence was developed to detect this new group of O3:K6 strains (16). While analyzing these O3:K6 strains recently isolated in India, Bag et al. observed the presence of a major ribotype, R4. By another PFGE method using *Not*I digestion, they showed the presence of one clone (1). The PFGE procedure used in this study was more discriminative, and the recently isolated O3:K6 strains could show eight different patterns.

In conclusion, our PFGE analysis has demonstrated that the new O3:K6 strains isolated after 1996 in Taiwan, Korea, Japan, and India form a genetically closely related group that is distinct from the O3:K6 and non-O3:K6 strains isolated before 1996. The results provide a line of evidence that the new clone of O3:K6 has caused a pandemic spread. However, the new O3:K6 strains and the earlier isolates did not differ in biological characteristics such as TDH production and susceptibilities to antibiotics and environmental stresses. A future study is needed to find out what other characteristic(s) is associated with the pandemicity of the clone.

ACKNOWLEDGMENTS

We thank the Department of Health of the Republic of China for financially supporting this research under contract no. DOH88-TD-1040.

REFERENCES

1. **Bag, P. K., S. Nandi, R. K. Bhadra, T. Ramamurthy, S. K. Bhattacharya, M. Nishibuchi, T. Hamabata, S. Yamasaki, Y. Takeda, and G. B. Nair.** 1999. Clonal diversity among recently emerged strains of *Vibrio parahaemolyticus* O3:K6 associated with pandemic spread. J. Clin. Microbiol. **37:**2354–2357.
2. **Beuchat, L. R.** 1975. Environmental factors affecting survival and growth of *Vibrio parahaemolyticus*. A review. J. Milk Food Technol. **38:**476–480.
3. **Centers for Disease Control and Prevention.** 1998. Outbreak of *Vibrio parahaemolyticus* infections associated with eating raw oysters—Pacific Northwest, 1997. Morb. Mortal. Wkly. Rep. **47:**457–462.
4. **Centers for Disease Control and Prevention.** 1999. Outbreak of *Vibrio parahaemolyticus* infection associated with eating raw oysters and clams harvested from Long Island Sound—Connecticut, New Jersey, and New York, 1998. JAMA **281:**603–604.
5. **Chiou, A., L.-H. Chen, and S.-K. Chen.** 1991. Foodborne illness in Taiwan, 1981–1989. Food Aust. **43:**70–71.
6. **Elliot, E. L., C. A. Kaysner, and M. L. Tamplin.** 1992. *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and other *Vibrio* spp., p. 111–140. *In* G. J. Jackson (ed.), Bacteriological analytical manual, 7th ed. AOAC International, Arlington, Va.
7. **Hasegawa, H., H. Hamashima, and T. Arai.** 1986. Mechanisms of plasmid-mediated antibiotic resistances in *Vibrio parahaemolyticus*. Microbiol. Immunol. **30:**437–444.
8. **Honda, T., Y. X. Ni, and T. Miwatani.** 1988. Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. Infect. Immun. **56:**961–965.
9. **Iguchi, T., S. Kondo, and K. Hisatsune.** 1995. *Vibrio parahaemolyticus* O serotypes from O1 to O13 all produce R-type lipopolysaccharide: SDS-PAGE and compositional sugar analysis. FEMS Microbiol. Lett. **130:**287–292.
10. **Johnson, D. E., L. Weinberg, J. Ciarkowski, P. West, and R. R. Colwell.** 1984. Wound infection caused by Kanagawa-negative *Vibrio parahaemolyticus*. J. Clin. Microbiol. **20:**811–812.
11. **Joseph, S. W., R. R. Colwell, and J. B. Kaper.** 1983. *Vibrio parahaemolyticus* and related halophilic vibrios. Crit. Rev. Microbiol. **10:**77–123.
12. **Jost, C., and M. G. Johnson.** 1978. Difference in injury of cells of *Vibrio parahaemolyticus* produced by heat and cold stresses in liquid and solid menstrual. J. Food Prot. **41:**764–767.
13. **Kelly, M. T., and E. M. Stroh.** 1989. Urease-positive, Kanagawa-negative *Vibrio parahaemolyticus* from patients and the environment in the Pacific Northwest. J. Clin. Microbiol. **27:**2820–2822.
14. **Koga, T., and K. Takumi.** 1995. Comparison of cross-protection against some environmental stresses between cadmium-adapted and heat-adapted cells of *Vibrio parahaemolyticus*. J. Gen. Appl. Microbiol. **41:**263–268.
15. **Martin-Kearley, J., J. A. Gow, M. Peloquin, and C. W. Greer.** 1994. Numerical analysis and the application of random amplified polymorphic DNA polymerase chain reaction to the differentiation of *Vibrio* strains from a seasonally cold ocean. Can. J. Microbiol. **40:**446–455.
16. **Matsumoto, C., J. Okuda, M. Ishibashi, M. Iwanaga, P. Garg, T. Rammamurthy, H. Wong, A. DePaola, Y. B. Kim, M. J. Albert, and M. Nishibuchi.** 2000. Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and *toxRS* sequence analyses. J. Clin. Microbiol. **38:**578–585.
17. **Mukhopadhyay, A. K., S. Garg, G. B. Nair, S. Kar, R. K. Ghosh, S. Pajni, A. Ghosh, T. Shimada, T. Takeda, and Y. Takeda.** 1995. Biotype traits and antibiotic susceptibility of *Vibrio cholerae* serogroup O1 before, during and after the emergence of the O139 serogroup. Epidemiol. Infect. **115:**427–434.
18. **Nishibuchi, M., and J. B. Kaper.** 1985. Nucleotide sequence of the thermostable direct hemolysin gene of *Vibrio parahaemolyticus*. J. Bacteriol. **162:**558–564.
19. **Okuda, J., M. Ishibashi, E. Hayakawa, T. Nishino, Y. Takeda, A. K. Mukhopadhyay, S. Garg, S. K. Bhattacharya, G. B. Nair, and M. Nishibuchi.** 1997. Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. J. Clin. Microbiol. **35:**3150–3155.
20. **Okuda, J., and M. Nishibuchi.** 1998. Manifestation of the Kanagawa phenomenon, the virulence-associated phenotype, of *Vibrio parahaemolyticus* depends on a particular single base change in the promoter of the thermostable direct haemolysin gene. Mol. Microbiol. **30:**499–511.
21. **Rudolph, K. M., A. J. Parkinson, and M. C. Roberts.** 1998. Molecular analysis by pulsed-field gel electrophoresis and antibiogram of *Streptococcus pneumoniae* serotype 6B isolates from selected areas within the United States. J. Clin. Microbiol. **36:**2703–2707.
22. **West, P. A., and R. R. Colwell.** 1984. Identification and classification of vibrionaceae—an overview, p. 285–363. *In* R. R. Colwell (ed.), Vibrios in the environment. John Wiley & Sons, New York, N.Y.
23. **Wong, H.-C., W.-L. Chao, and S.-J. Lee.** 1990. Incidence and characterization of *Listeria monocytogenes* in foods available in Taiwan. Appl. Environ. Microbiol. **56:**3101–3104.
24. **Wong, H.-C., and Y. S. Lee.** 1994. Regulation of iron on growth and production of thermostable direct hemolysin by *Vibrio parahaemolyticus* in intraperitoneal infected mice. Microbiol. Immunol. **38:**367–371.
25. **Wong, H.-C., K.-T. Lu, T.-M. Pan, C.-L. Lee, and D. Y. C. Shih.** 1996. Subspecies typing of *Vibrio parahaemolyticus* by pulsed-field gel electrophoresis. J. Clin. Microbiol. **34:**1535–1539.
26. **Wong, H.-C., P. Y. Peng, J. M. Han, C. Y. Chang, and S. L. Lan.** 1998. Effect of mild acid treatment on the survival, enterotoxigenicity, and protein production in *Vibrio parahaemolyticus*. Infect. Immun. **66:**3066–3071.